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PRINCIPAL INVESTIGATOR: Kristine S. Vogel, Ph.D.

CONTRACTING ORGANIZATION: University of Texas Health Science Center at San Antonio
San Antonio, Texas 78229

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Kristine S. Vogel, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas Health Science Center at San Antonio
San Antonio, Texas 78229

E-Mail: vogelk@uthscsa.edu

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13. ABSTRACT *(Maximum 200 Words)*

Our purpose is to examine the role of the NF1 gene product, neurofibromin, in modulating the migratory and invasive properties of neural crest cells (NCC) and neural crest-derived sarcoma cells. As a negative regulator of Ras signaling, neurofibromin may influence the responses of NC-derived cells to growth factors and extracellular matrix (ECM) molecules that affect motility. We use embryonic NCC and NC-derived sarcoma lines isolated from cisNf1;p53 mice to compare integrin ECM receptor expression patterns, ECM adhesion preferences, migration on ECM substrata, invasion through ECM barriers, and dispersal along NCC pathways in vivo for wild-type and neurofibromin-deficient cells. In the five months since funding was transferred to UTHSCSA, we have developed dissection and culture methods for embryonic mouse branchial arch neural crest cells, and determined that Nf1-/- maxillary and mandibular NCC are more invasive through fibronectin and laminin. In addition, we have correlated MTT phenotype with invasive potential, and characterized effects of growth factors on MTT sarcoma cell invasiveness. Our studies address two important questions: 1) what molecules control the migration and localization of NCC in the embryo? 2) which growth factor signaling pathways affect the invasiveness of NC-derived sarcoma cells?

14. SUBJECT TERMS
neurofibromatosis type 1; neural crest cells; cell motility and Migration; extracellular matrix receptors; MTT; neurofibromin

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INTRODUCTION

The neural crest of vertebrate embryos is a migratory population of stem cells that give rise to sensory and autonomic neurons, Schwann cells, melanocytes, and smooth muscle cells in the outflow tract of the heart (Weston, 1991). Mice harboring targeted null mutations in the Nf1 and p53 tumor suppressor genes in the cis configuration spontaneously develop malignant soft tissue sarcomas derived from the neural crest (Cichowski et al., 1999; Vogel et al., 1999). These mice provide a model for malignancies (malignant peripheral nerve sheath tumors, malignant Triton tumors) associated with neurofibromatosis type 1, an autosomal dominant neoplastic disorder that primarily affects neural crest derivatives. The objectives of the proposed research are 1) to analyze the effects of Nf1 gene dosage on the migratory and invasive properties of primary neural crest cells isolated from E9-E11.5 mouse embryos, and 2) to correlate integrin expression patterns and Ras activation with invasive potential in neural crest-derived sarcoma lines isolated from cisNf1+/-;p53+/- mice. In the 5 months since funds were transferred to UTHSCSA from LSUMC, we have developed methods to isolate and culture branchial arch NCC, and begun to assay invasiveness of these primary embryonic cells (Tasks 1 and 2). We have continued in vitro adhesion and invasiveness assays with the MTT sarcoma lines, and demonstrated differential effects of bFGF (basic fibroblast growth factor) and TGFβ1 (transforming growth factor) treatment on invasiveness through collagen IV, fibronectin, and laminin matrices. In addition, we isolated four new glioma cell lines from a cisNf1+/-;p53+/- mouse, to provide CNS-derived astrocytic lines as a comparison for PNS-derived Schwann cell lines.

BODY

Task 1. Compare NCC dispersal in wild-type and Nf1-/- mouse embryos

Note: For revised statement of work, and following transfer of funds from LSUHSC, Month 1 = June 2001

- **Analyze melanocyte and neuronal precursor dispersal patterns (Months 6-18)**
- **Analyze dispersal of NCC in vitro on ECM substrata and in presence of growth factors (Months 0-24)**
- **Analyze dispersal of mouse NCC on medial and lateral migration pathways in vivo following transplant (Months 12-36)**

Results for Task 1, June 2001-October 2001

To begin to analyze dispersal patterns of mouse NCC in vitro, we developed techniques to isolate branchial arch mesenchyme from E10, E11, and E11.5 mouse embryos, and to maintain these neural crest-derived cells in Neurobasal medium supplemented with B27, fetal calf serum, and GlutaMax (all from Gibco). Using electrolytically-sharpened tungsten needles, we microdissected branchial arches from the progeny of Nf1+/- matings; the maxillary and mandibular processes (Branchial Arch 1) and the hyoid arch (Branchial Arch 2) were dissected from both sides of each embryo. Initially, we allowed branchial arches to attach directly to tissue culture plastic, and subsequently tried plating branchial arch explants onto glass coverslips coated with polyornithine and either fibronectin or laminin. We found that explants, regardless of genotype, did not attach to ECM substrata in a consistent manner, and thus NCC will have to be dissociated from tissue culture plastic and replated onto ECM substrata for migration studies (see Task 2). It is possible that the non-neural crest branchial arch epithelia inhibit attachment; these epithelia can be removed by brief trypsin treatment and microdissection, prior to plating the explant. Explants of trunk neural tube and neural crest from E9.5 mouse embryos attach to ECM substrata in a consistent manner (K.S. Vogel, unpublished observations), and thus may be better suited for dispersal studies.

Task 2. Compare expression of integrin ECM receptors and motile characteristics for wild-type and Nf1-/- mouse NCC

- **Perform in vitro migration assays for embryonic NCC on ECM substrata (Months 12-24)**
- **Perform in vitro invasiveness and chemotaxis assays for embryonic NCC through ECM substrata (Months 6-18)**
- **RT-PCR for integrin and RHAMM ECM receptors in embryonic NCC (Months 12-24).**

Results for Task 2, June 2001-October 2001

We found that branchial arch neural crest cells isolated from E10-E11.5 mouse embryos will attach to tissue culture plastic and proliferate extensively in Neurobasal medium containing B27, 5% FCS, and GlutaMax. Both ectodermally- and endodermally-derived branchial arch epithelia can be removed from the cultures using a thin blunt glass probe. With branchial arch NCC isolated from the E11.5 progeny of an Nf1+/- mating, we performed invasiveness and chemotaxis assays on three different NCC populations from Nf1+/+, Nf1+/-, and Nf1-/- embryos (**Figures 1 and 2**). **Figure 1** shows relative invasiveness of NCC isolated from maxillary, mandibular, and hyoid arches through fibronectin (10 μg/ml), with 2% fetal calf serum as a chemoattractant. For both branchial arch 1 populations (maxillary and mandibular processes), cells derived from the Nf1-/- embryo exhibited increased invasive ability, when compared to Nf1+/- and Nf1+/+ cells. Hyoid arch NCC with different Nf1 gene dosages did not display consistent differences in invasiveness through fibronectin matrices. **Figure 2** shows the results of invasiveness assays through laminin (10 μg/ml) matrices, using the same branchial arch NCC populations and 2% FCS as a chemoattractant. Again, Nf1-/- arch 1 (maxillary and mandibular processes) neural crest cells exhibited increased invasive potential, although hyoid arch cells displayed no consistent differences between genotypes. We are currently repeating these experiments with branchial arch neural crest cells isolated from E10, E10.5, E11, and E11.5 mouse embryos, to identify possible temporal differences in invasive potential. Trunk neural crest cells, isolated from E9.5-E10 mouse embryos, will be compared in the same transwell invasiveness assay system. Now that our transwell assay is working consistently for primary mouse NCC, we also plan to test invasiveness through collagen IV matrices, concomitant with the fibronectin and laminin assays.

Since neural crest cells migrate extensively throughout the head and trunk of the embryo, we reasoned that these cells might be especially invasive, when compared to other potentially motile embryonic cell types. Therefore, we prepared cultures of mouse embryo fibroblasts (MEFs) from the E12.0 progeny of an Nf1+/- mating, and challenged these cells with collagen IV, fibronectin, and laminin matrices in transwell invasiveness assays. Preliminary results indicate that, although Nf1-/- and Nf1+/- MEFs are more invasive through collagen IV matrices than are Nf1+/+ MEFs, the fibroblasts (presumably mesoderm-derived) are much less invasive than neural crest cells isolated from similar-stage embryos. The MEFs were given 6 additional hours to invade through matrices, and average numbers of cells per field did not exceed 10, even for Nf1-/- cells (data not shown).

Task 3. Correlate integrin expression, cell adhesion, and responses to TGFβ1 with invasive and metastatic ability in mouse MTT sarcoma lines

- **Compare adhesion of MTT sarcoma lines to ECM substrata (Months 0-12)**
- **Perform RT-PCR and Western blot analyses of integrin expression in MTT sarcoma lines (Months 0-12)**
- **Perform in vitro migration and invasiveness assays on hyaluronan (Months 12-24)**

- **Analyze MTT sarcoma cell invasiveness in micropore filter (transwell) assays (Months 0-18)**
- **Perform in vivo NCC pathway migration assays with MTT sarcoma lines (Months 0-36)**

Results for Task 3, June 2001-October 2001

1. Adhesion of MTT sarcoma lines to ECM substrata. Dr. Thomas Lallier, our collaborator at LSUHSC School of Dentistry, has chosen to focus on sarcoma lines isolated from tumor Tu8, which developed in a cisNf1+/-;p53+/- mouse in the frontonasal region. Tu8 clonal lines express low levels of Schwann cell and smooth muscle traits, and do not express neuronal traits. Both Tu8-5 and Tu8-9 respond to basic fibroblast growth factor (bFGF) treatment with increased invasiveness in vitro (see **Figure 7**). **Figures 3 and 4** show results of adhesion assays with Tu8 sarcoma lines and a variety of ECM substrata (fibronectin, vitronectin, laminin, collagens type I and IV, and the RGD peptide) at two different time points.
2. Western blot analyses of integrin expression in MTT sarcoma lines. To date, we have optimized conditions for immunoblot analysis of two integrin subunits, α5 and αV (both antibodies from Chemicon). **Table 1** summarizes the results of experiments designed to determine whether bFGF or TGFβ1 treatment can affect levels of α5 or αV integrin expression. All MTT cell lines tested to date express αV integrin, but levels of expression do not appear to be affected by growth factor treatment. We are currently attempting to correlate α5 expression with motility and invasiveness on fibronectin substrata, particularly for the non-invasive line 61E9.

Table 1

MTT Cell Line	Growth Factor	α5 Expression	αV Expression
61E9	None	-	++
	bFGF	+	++
	TGFβ1	+	++
Tu9-8	None	+	++
	bFGF	+	++
	TGFβ1	++	++
Tu19-1	None	++	++
	bFGF	+	++
	TGFβ1	+	++
Tu19-10	None	+	++
	bFGF	++	++
	TGFβ1	-	+++
Tu26-3	None	-	+++
	bFGF	+	+++
	TGFβ1	++	+++
Tu26-6	None	++	++
	bFGF	++	++
	TGFβ1	++	++

3. Invasiveness of MTT cell lines in micropore filter (transwell) assays. While optimizing conditions for in vitro transwell assays, we noticed that the serum concentration (10% vs. 2%) of the medium that sarcoma cells are maintained in affects both invasiveness and

response to serum as a chemoattractant. For all invasiveness assays, 5000 cells are plated on the matrix-coated top surface of each transwell (Costar), in serum-free DMEM. **Figure 5** compares the invasiveness of three sarcoma lines, isolated from the same primary tumor, with that of a MEF line that also lacks neurofibromin and p53, through collagen IV (1 μg/ml), fibronectin (10 μg/ml), and laminin (10 μg/ml) matrices. Unlike most MTT sarcoma lines, Tu19-1 and Tu19-3 express numerous neuronal traits, whereas Tu19-10 expresses low levels of some neuronal traits (K.S. Vogel, unpublished data). Tu19-1 and Tu19-3 are highly invasive through matrices of all 3 molecules, especially when maintained in 10% HIFCS medium prior to assay (**Figure 5**). Tu19-10 exhibits more limited invasiveness through collagen IV and fibronectin, and the MEF cell line is less invasive than the neural crest-derived sarcomas.

a) Growth factor effects on MTT invasiveness. Both bFGF and TGFβ1 can alter the invasive potential of a variety of tumor cell types (Hamel and Westphal, 2000; Pasche et al., 2001). However, the effects of these growth factors on the motile behavior of neural crest-derived sarcoma cells, particularly in the absence of neurofibromin function, have not been examined. For all growth factor experiments, tumor cell lines are maintained in 2% HIFCS/DMEM during treatment (3-5 days), prior to transwell assay. **Figure 6** shows that for the neuronal line Tu19-1, bFGF (5 ng/ml) treatment enhances invasiveness through both fibronectin and collagen matrices, whereas TGFβ1 (5 ng/ml) treatment inhibits invasiveness. **Figure 7** compares effects of bFGF and TGFβ1 treatment on the invasiveness of two MTT lines, isolated from the same facial sarcoma, through fibronectin and laminin matrices. Although TGFβ1 does not have a significant effect on the invasiveness of these two sarcoma lines, bFGF increases invasiveness through fibronectin for both lines, and through laminin for Tu8-5. **Figure 8** compares invasiveness and growth factor responsiveness of a neuronal sarcoma line, Tu19-3, and a differentiated MTT line, Tu26-6, that expresses high levels of both smooth muscle and Schwann cell traits. Both cell lines respond to bFGF treatment with increased invasiveness through fibronectin and laminin, and TGFβ1 treatment decreases invasiveness for Tu19-3. The invasiveness of two MTT sarcoma lines in the absence of a chemoattractant is compared in **Figure 9**. Although 61C8 responds to TGFβ1 treatment with decreased invasiveness, it does not appear to respond to bFGF. Tu26-3, another highly differentiated MTT line, responds to both growth factors with changes in invasiveness. **Figure 10** shows invasiveness of a "control" fibrosarcoma line, Tu39, isolated from an Nf1+/+;p53-/- mouse. This line does not express neural crest traits, exhibits low invasiveness overall, and does not respond significantly to bFGF or TGFβ1 with changes in invasiveness. Our results to date indicate that 1) neuronal lines are more invasive than lines expressing only Schwann cell and/or smooth muscle traits, 2) TGFβ1 treatment inhibits the invasiveness of cisNf1;p53 sarcoma lines, particularly those that express neuronal traits, 3) bFGF enhances invasiveness for many cisNf1;p53 sarcoma lines.

b) Invasiveness of cisNf1;p53 glioma cell lines. CisNf1+/-;p53+/- mice develop CNS gliomas, in addition to the soft tissue sarcomas derived from PNS neural crest precursors (Reilly et al., 2000). We isolated four clonal lines from one such tumor, to provide comparisons for the invasive and motile behavior of lines derived from PNS Schwann cell precursors. In contrast to Schwann cell-derived tumors, the invasive behavior and growth factor responsiveness of malignant gliomas has been studied extensively (Ashley et al., 1998; Monaghan et al., 2000; Mori et al., 2000; Platten et al., 2000). **Figure 11** shows initial results for the invasiveness of two glioma cell lines, Tu96i-1 and Tu96i-4, through collagen IV, fibronectin, and laminin

matrices, both with and without serum as a chemoattractant. Tu96i-4 in particular is highly invasive in response to the serum chemoattractant, through all three matrix molecules.

Task 4. Analyze role of Ras signaling in locomotory, invasive, and metastatic properties of mouse MTT sarcoma lines

- **Compare Ras activation levels in MTT sarcoma lines with different invasiveness properties (Months 18-36)**
- **Determine Rac and Rho activation levels in MTT sarcoma lines (Months 24-36)**
- **Block function of Ras and other small GTPases to determine effect on MTT sarcoma cell motility and invasiveness (Months 18-36)**

Figure 1.
Neural Crest Cell Invasiveness Through Fibronectin

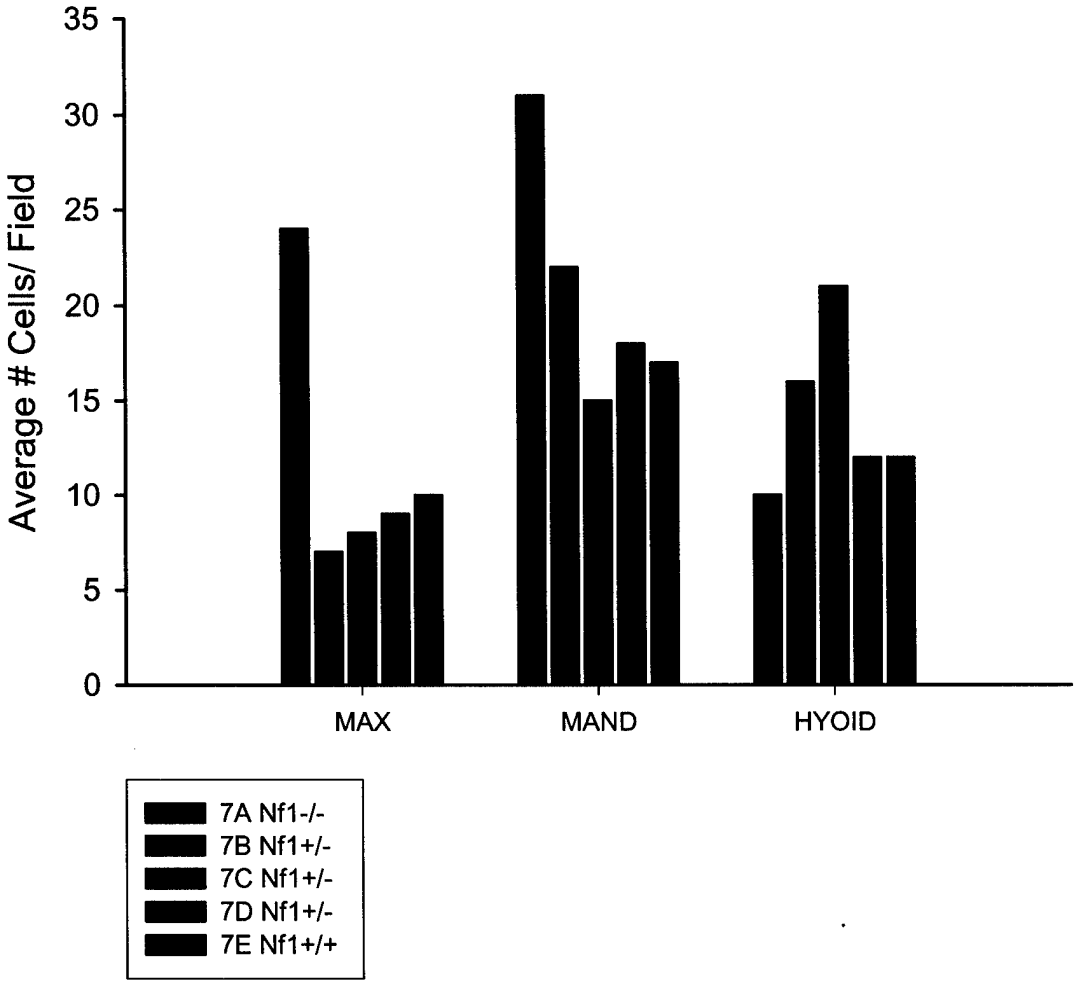

Figure 2.
Neural Crest Cell Invasiveness Through Laminin

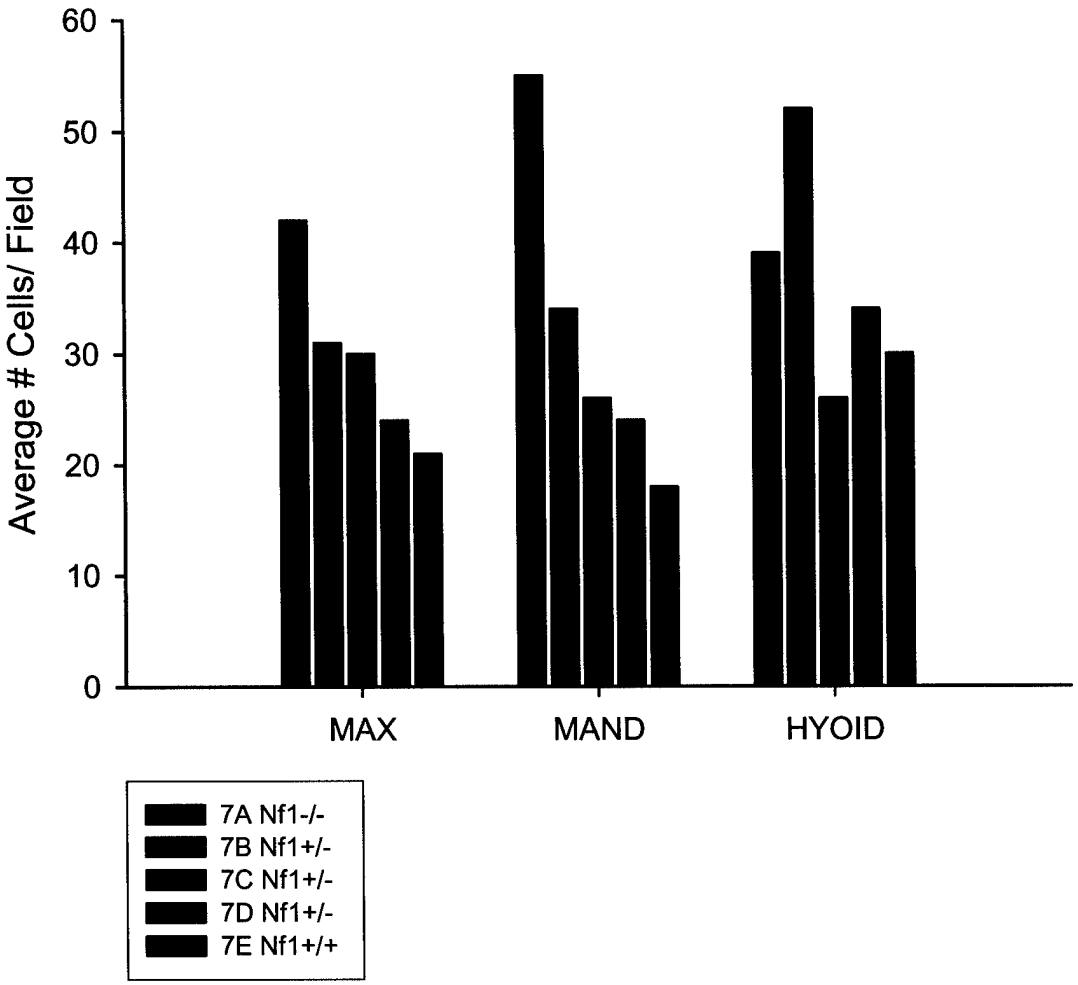

Figure 3. Tumor Cell Line Adhesion 30 minutes

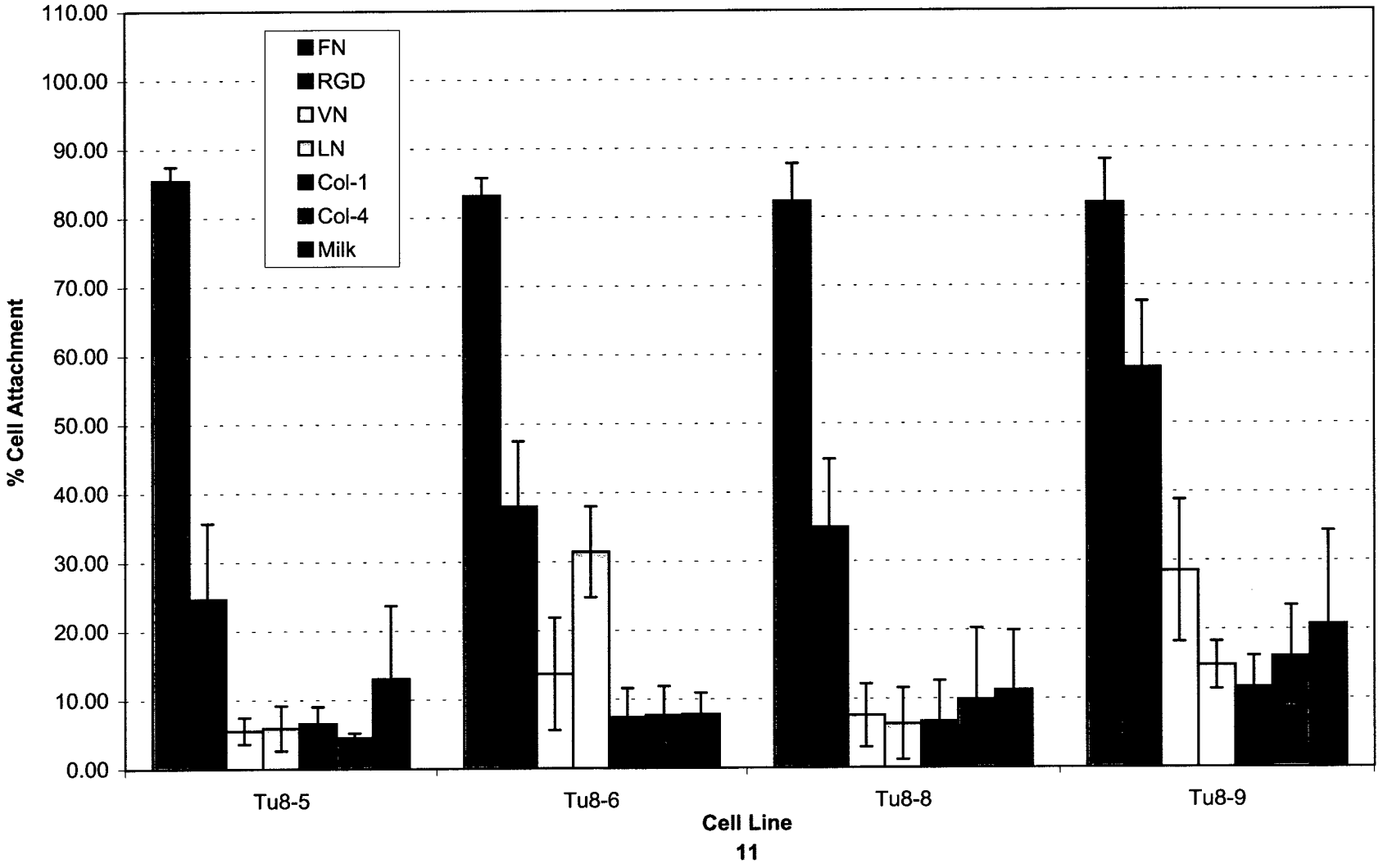

Figure 4. Tumor Cell Line Adhesion 60 minutes

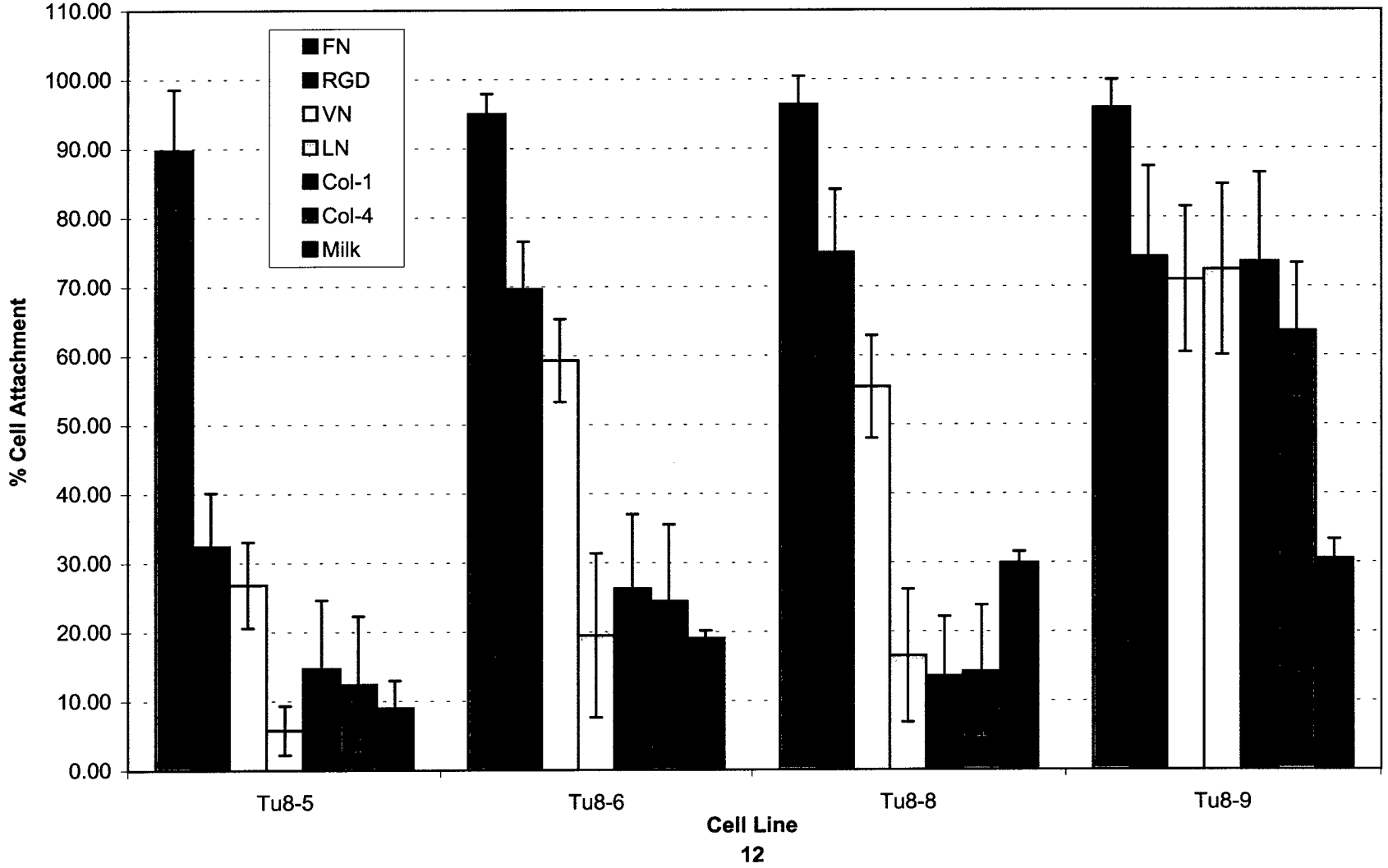

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Figure 5.
Invasiveness Through ECM
cisNf1;p53 Sarcoma Lines Tu19-1, Tu19-3, Tu19-10
Nf1-/-;p53-/- MEF Line 63C

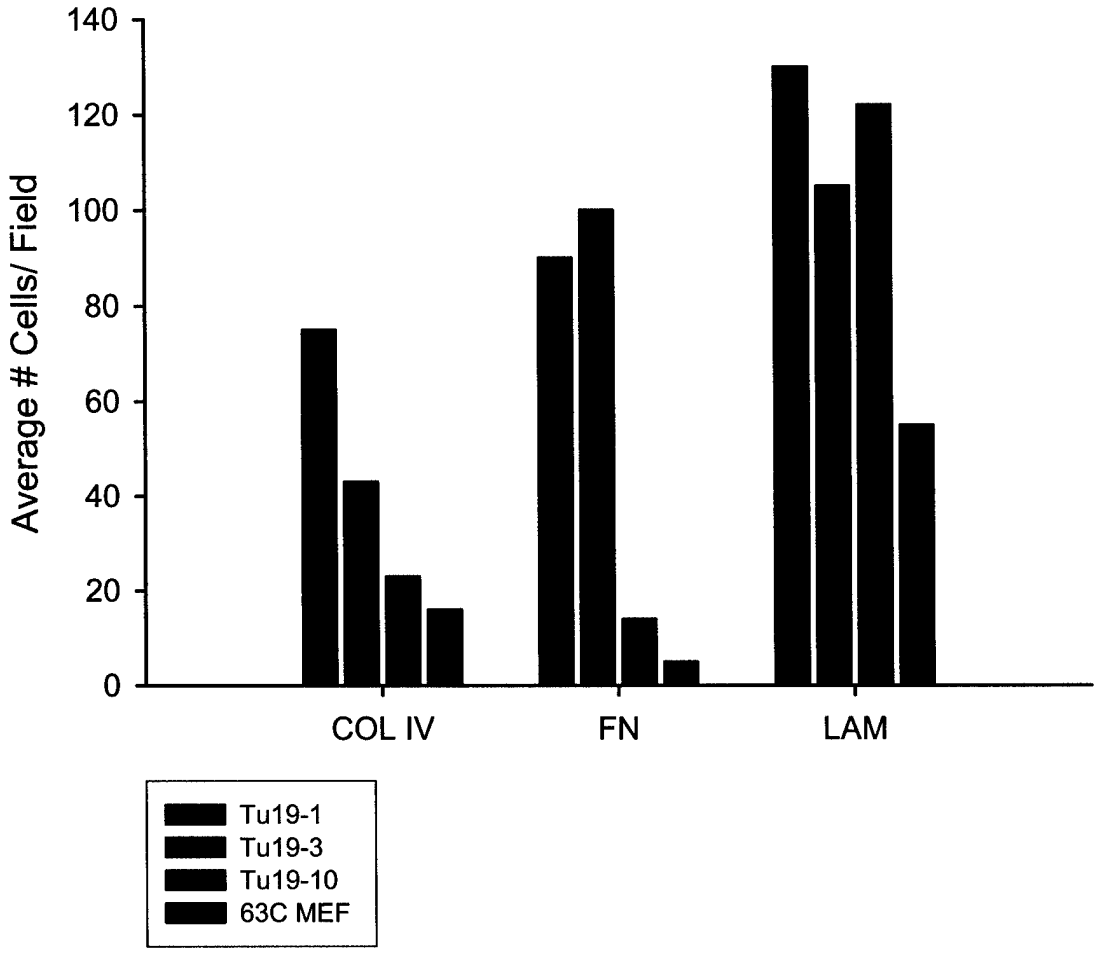


Figure 6.
Growth Factor Effects on Invasiveness
2% HIFCS as Chemoattractant
cisNf1;p53 Sarcoma Line Tu19-1

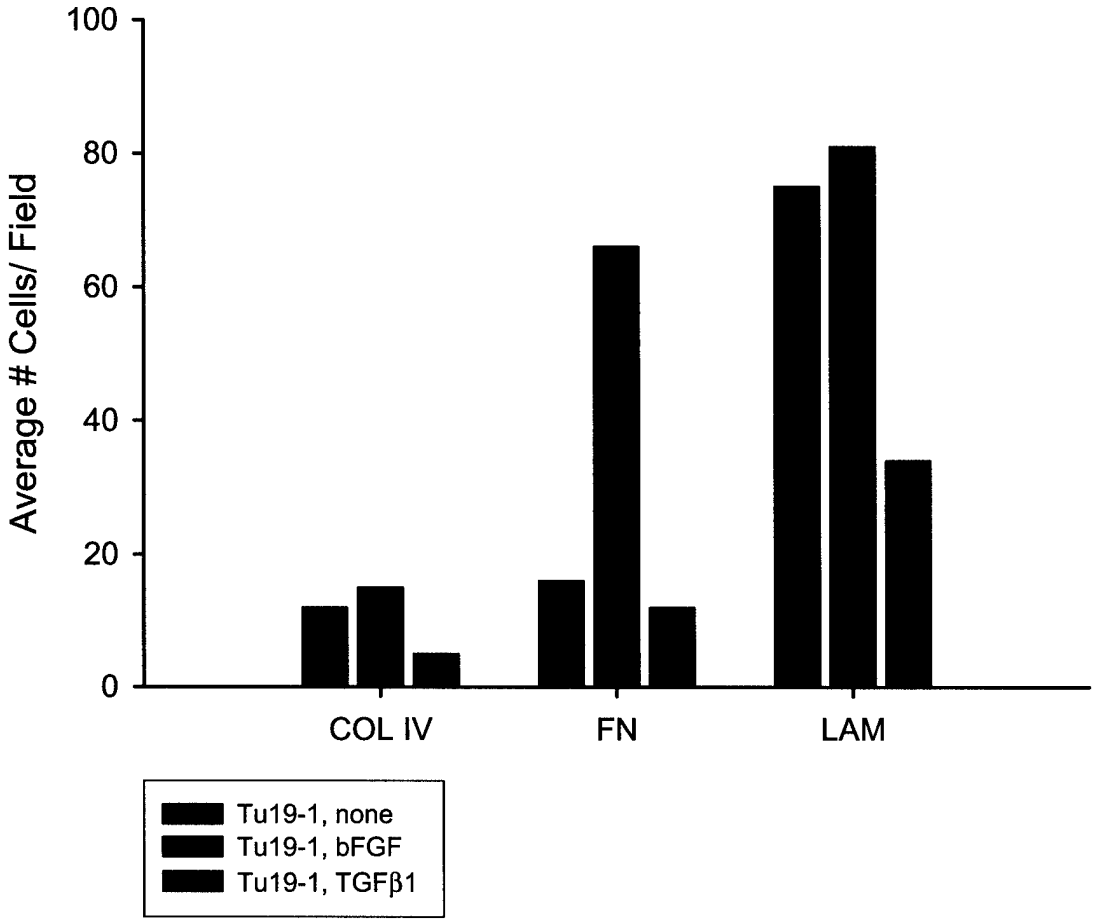


Figure 7.
Growth Factor Effects on Invasiveness
2% HIFCS as Chemoattractant
cisNf1;p53 Sarcoma Lines Tu8-5 and Tu8-9

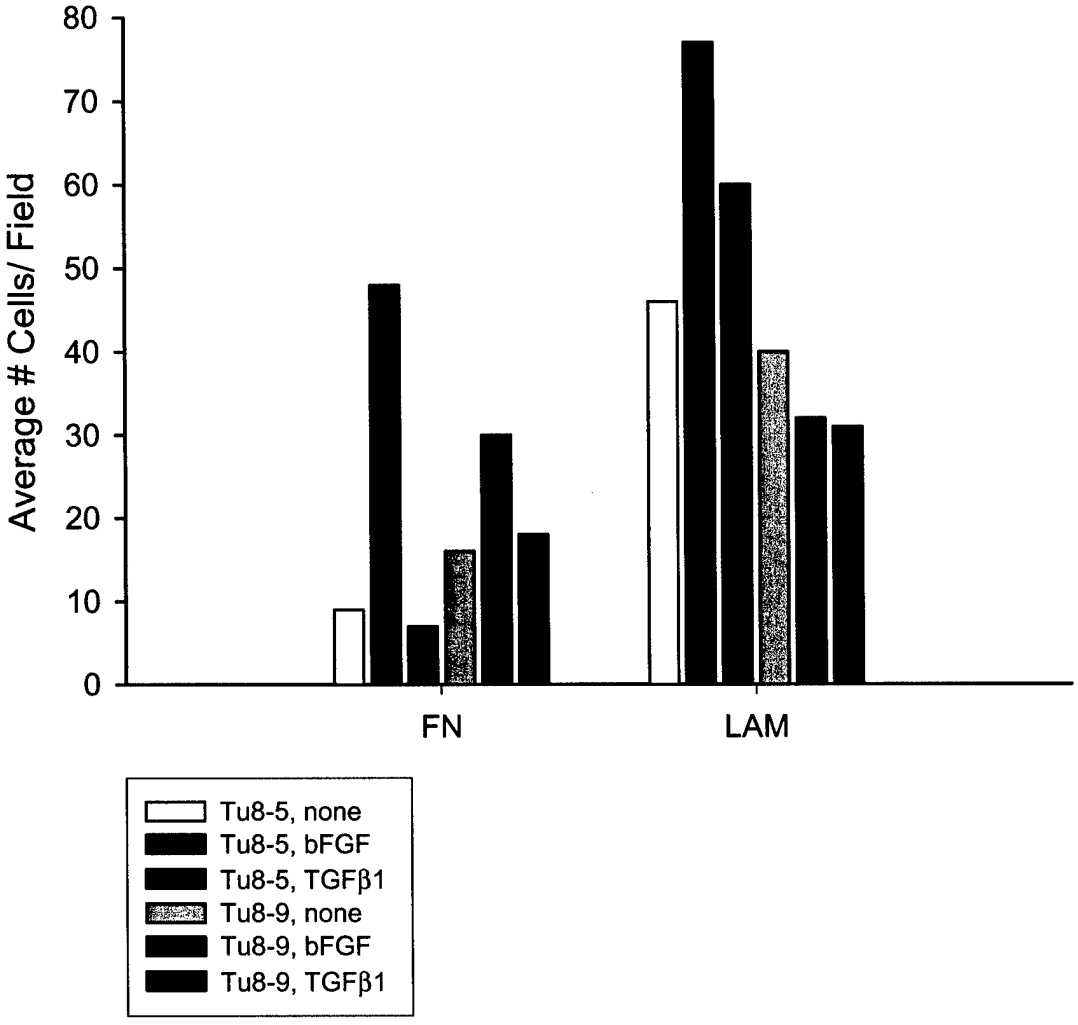


Figure 8.
Growth Factor Effects on Invasiveness
2% HIFCS as Chemoattractant
cisNf1;p53 Sarcoma Lines Tu19-3 and Tu26-6

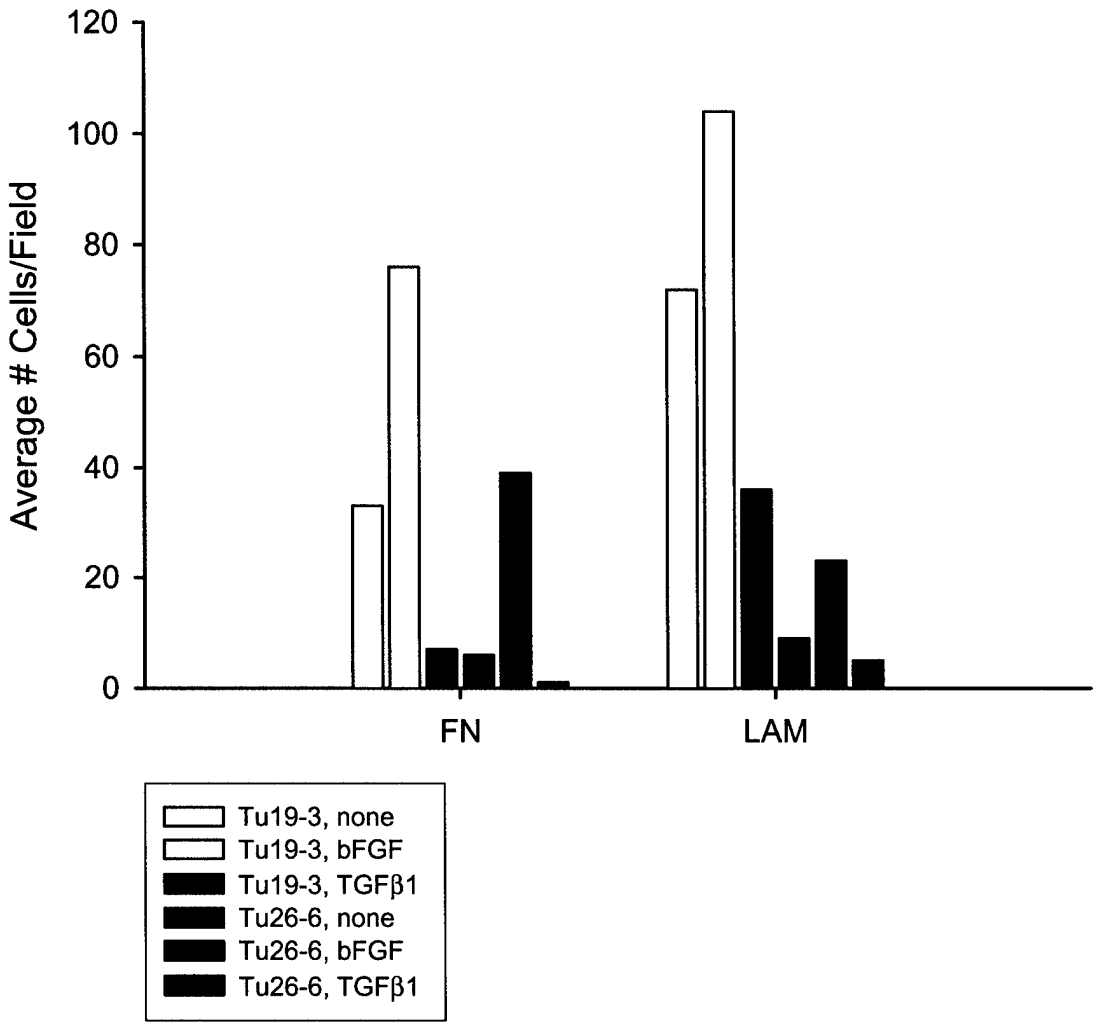


Figure 9.
Growth Factor Effects on Invasiveness
No Chemoattractant
cisNf1;p53 Sarcoma Lines 61C8 and Tu26-3

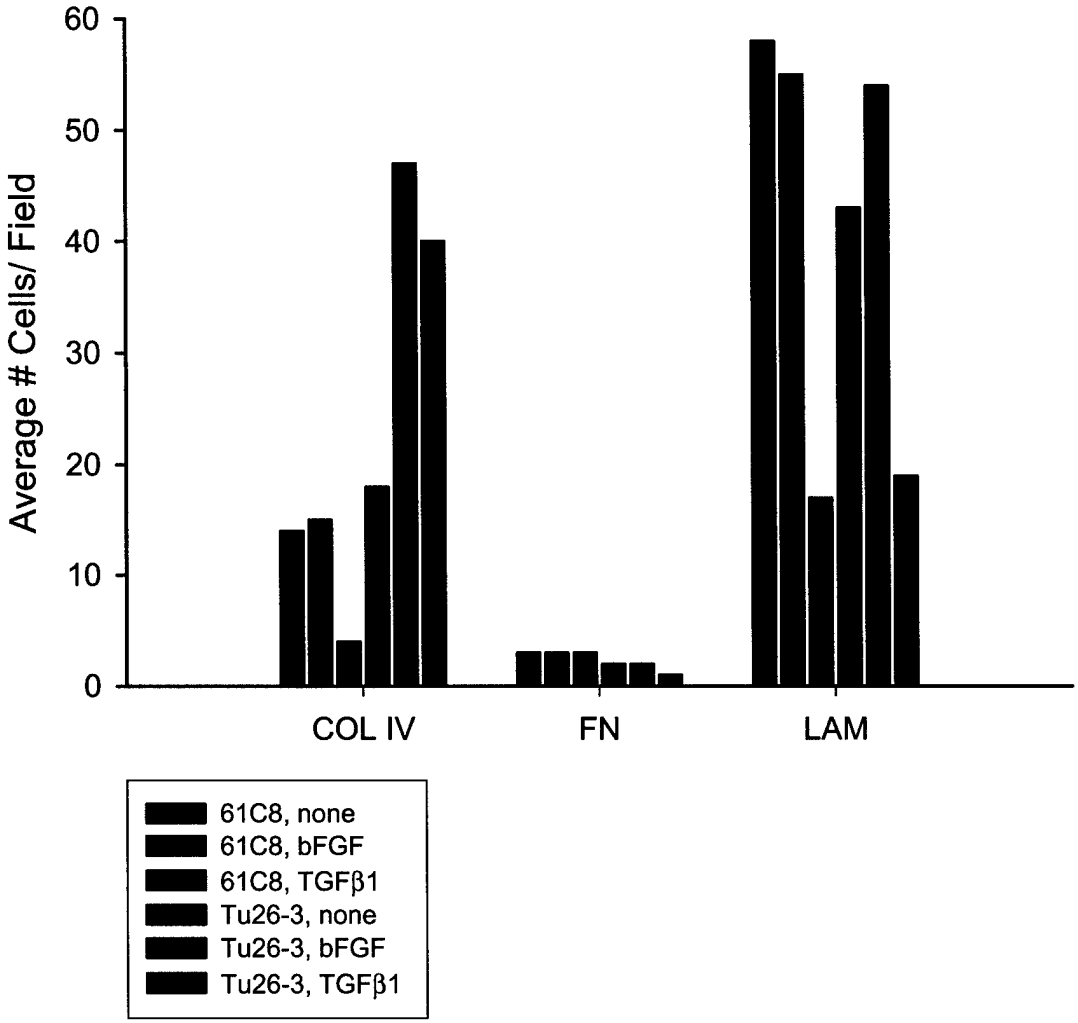


Figure 10.
Growth Factor Effects on Invasiveness
No Chemoattractant
Nf1+/+; p53-/- Fibrosarcoma Line Tu39

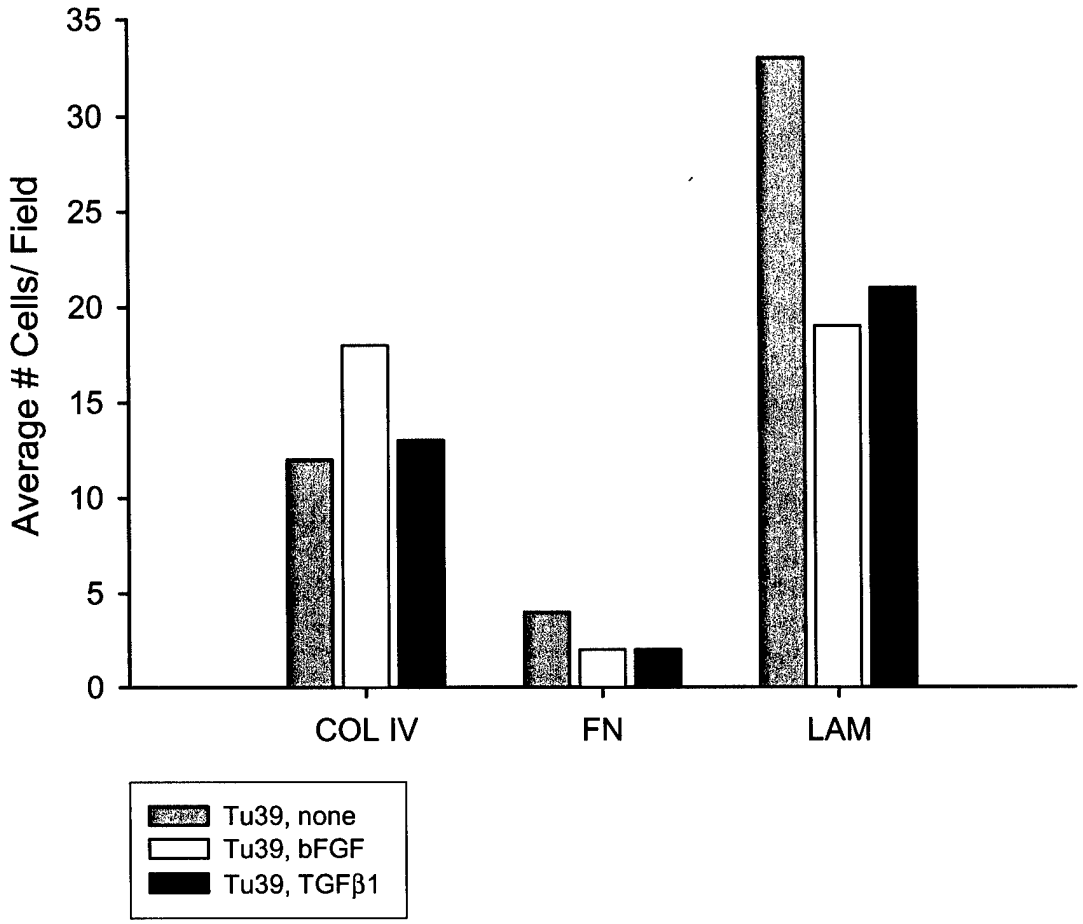
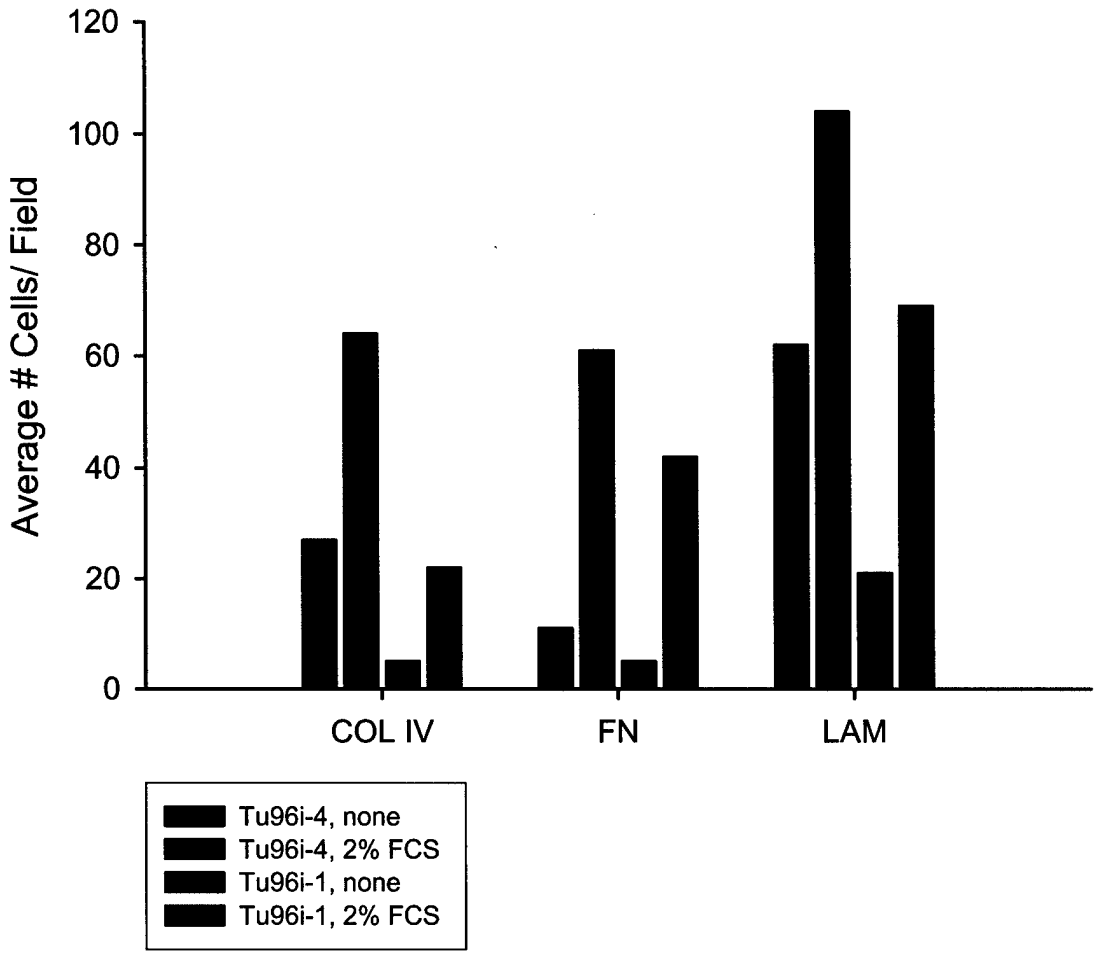


Figure 11.
Invasiveness through ECM
cisNf1;p53 Glioma Cell Lines



KEY RESEARCH ACCOMPLISHMENTS

June 2001-October 2001

Tasks 1 and 2

- Developed microdissection and culture protocols for E10-E11.5 mouse branchial arch neural crest cells (maxillary, mandibular, hyoid)
- Optimized in vitro invasiveness assay for branchial arch neural crest cells
- Performed initial in vitro invasiveness assays with branchial arch NCC isolated from E11.5 Nf1+/+, Nf1+/-, and Nf1-/- mouse embryos

Task 3

- Continued cell adhesion assays with Tu8 MTT line
- Optimized Western blot conditions for α5 and αV integrins
- Compared effects of bFGF and TGFβ1 treatment on α5 and αV integrin expression for MTT cell lines
- Optimized in vitro invasiveness assay through collagen IV, fibronectin, and laminin matrices for MTT sarcoma lines
- Compared effects of bFGF and TGFβ1 treatment on invasiveness for MTT sarcoma lines
- Isolated 6 new glioma lines (4 clonal) from cisNf1+/-;p53+/- mice
- Began to assay invasiveness of clonal glioma lines

Task 4

REPORTABLE OUTCOMES

1. **Presentations**

a) 1-25-01 Seminar, Pediatrics Department, UTHSCSA "A mouse model for malignancies associated with NF1"

b) Abstract, "Motility and Invasiveness of Novel Neural Crest-Derived Sarcoma and malignant Glioma Cell Lines Isolated from cisNf1+/-;p53+/- Mice" K.S.Vogel, Center for Biomedical Neurosciences Meeting, UTHSCSA

2. **Development of Cell Lines**

a) 8 new cisNf1;p53 neural crest-derived sarcoma lines

b) 6 new cisNf1;p53 glioma lines

c) 6 Nf1 mouse embryo fibroblast lines

3. **Funding Applied For Based on Work Supported by Award**

a) Dental Student Research Fellowship, UTHSCSA, June 2001-August 2001, "Role of Neurofibromin in Regulating Cranial Neural Crest Cell Proliferation and Migration"-award provided research stipend for Taylor Starr, dental student

b) PENDING: DoD NFRP IDEA Award proposal "Neurofibromin and Neuronal Apoptosis"

4. **Employment and Research Opportunities**

a) Maria Kanter, Ph.D., research scientist

b) Marisa Lopez-Cruzan, graduate student rotation project

c) Christopher Geyer, graduate student rotation project

d) Taylor Starr, Dental Student Research Fellowship

CONCLUSIONS

Tasks 1 and 2. Our initial experiments indicate that loss of neurofibromin function may increase invasiveness of primary NCC, at least for maxillary and mandibular branchial arch populations. We are repeating these experiments with branchial arch neural crest cells isolated

from E10, E10.5, E11, and E11.5 embryos, to determine if there are temporal changes in NCC invasiveness. Since the neural crest-derived MTT lines studied in Task 3 lack both neurofibromin and p53 (Vogel et al., 1999), we plan to examine the invasive and motile behavior of neural crest cells isolated from Nf1-/-;p53-/- mouse embryos, which comprise 25% of the embryos in litters from cisNf1+/-;p53+/- matings prior to E13. Second-year UTHSCSA dental student Taylor Starr is assisting with these experiments, and will present our results at the American Association for Dental Research meeting in spring 2002.

A recent paper (Haack and Hynes, 2001) reports the results of experiments in which α4 or α5 integrin-deficient mouse NCC were transplanted to the neural crest migration pathway of chick embryos to monitor effects on migratory potential. We are encouraged that similar experiments with neurofibromin-deficient mouse NCC will yield important and relevant information about the role of this molecule in neural crest migration and localization during embryonic development.

Task 3. We have continued our integrin expression, cell adhesion, and invasiveness assays with neurofibromin-deficient MTT sarcoma lines. Although the expression of integrins in peripheral neuropathies, nerve injury paradigms, and neuroblastomas has been reported, the expression and roles of these ECM receptors are largely uncharacterized in neural crest-derived nerve sheath tumors (MPNST, MTT; Previtali et al., 2001). Our mouse model and representative cell lines for these tumors provide a unique opportunity to correlate integrin expression, cell motility, phenotype, and invasiveness in the context of neural crest-derived sarcomas. Our results to date indicate that cisNf1;p53 sarcoma lines that express neuronal traits (Tu19 lines) are more invasive, particularly through collagen IV and fibronectin matrices, than are MTT lines that express only Schwann cell and smooth muscle traits. Moreover, differentiated cell lines that express high levels of Schwann cell and smooth muscle traits are less invasive than their relatively poorly differentiated counterparts. These results may be applicable to the assessment of invasive potential of human peripheral nerve sheath tumors.

During our analyses of growth factor effects on MTT neural crest phenotype, we noticed that both bFGF and TGFβ1 altered sarcoma cell morphology and proliferation. Moreover, these factors have well-characterized effects on the motile, adhesive, and invasive characteristics of a variety of tumor cell types. To begin to characterize the specific effects of bFGF and TGFβ1 on the invasiveness of neural crest-derived sarcomas, we exposed MTT cell lines to these growth factors, and then challenged the cells with in vitro invasiveness assays. Our results to date indicate that, for most MTT sarcoma lines, bFGF treatment enhances invasiveness and TGFβ1 treatment inhibits invasiveness. Some cell lines are not responsive to one or both factors. Therefore, we propose to examine expression of bFGF and TGFβ1 receptors in the MTT sarcoma lines by Western blot analysis. In addition, we propose to examine activation of downstream signaling pathways (Ras, MAPK, PI3 kinase, Smads) in the MTT lines, again using Western blot analyses (would be included under Task 4). Finally, we are confident that the chick neural crest migration pathway transplant technique is the "gold standard" for assessing the motile properties of neural crest-derived cells, and we are continuing these experiments at UTHSCSA.

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